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Sestrins Function as Guanine Nucleotide Dissociation Inhibitors for Rag GTPases to Control mTORC1 Signaling

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SUMMARY

Mechanistic target of rapamycin complex 1 (mTORC1) integrates diverse environmental signals to control cellular growth and organismal homeostasis. In response to nutrients, Rag GTPases recruit mTORC1 to the lysosome to be activated, but how Rags are regulated remains incompletely understood. Here we show that Sestrins bind to the heterodimeric RagA/B-RagC/D GTPases, and function as guanine nucleotide dissociation inhibitors (GDIs) for RagA/B. Sestrin overexpression inhibits amino acid-induced Rag guanine nucleotide exchange and mTORC1 translocation to the lysosome. Mutation of the conserved GDI motif creates a dominant-negative form of Sestrin that renders mTORC1 activation insensitive to amino acid deprivation, whereas a cell-permeable peptide containing the GDI motif inhibits mTORC1 signaling. Mice deficient in all Sestrins exhibit reduced postnatal survival associated with defective mTORC1 inactivation in multiple organs during neonatal fasting. These findings reveal a non-redundant mechanism by which the Sestrin family of GDIs regulates the nutrient-sensing Rag GTPases to control mTORC1 signaling.

INTRODUCTION

Mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of cellular growth, whose dysregulation results in a plethora of pathological conditions including cancer, diabetes, and aging (Laplante and Sabatini, 2012). mTORC1 regulates macromolecule biosynthesis and energy metabolism by integrating intracellular and environmental signals such as growth factors and nutrients that impinge on mTORC1 signaling through the Rheb and Rag subfamilies of GTPases (Bar-Peled and Sabatini, 2014; Dibble and Manning, 2013; Jewell et al., 2013). As small GTPases, Rheb and Rags function on endomembranes to regulate mTORC1 signaling (Betz and Hall, 2013). While Rheb is targeted to the endomembranes via a lipid-binding motif (Saito et al., 2005), Rags are

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AUTHOR CONTRIBUTIONS

M.P. and M.O.L. conceived the project. M.P. designed and performed most experiments with input from M.O.L. N.Y. performed experiments. M.P. and M.O.L. analyzed the data. M.P. wrote and M.O.L. edited the manuscript.

anchored to the lysosomal surface by the Ragulator complex (Sancak et al., 2010). In addition, Rags function as obligate heterodimers with RagA or the highly related RagB binding to RagC or RagD that are also homologous to each other (Kim et al., 2008; Sancak et al., 2008; Sekiguchi et al., 2001). Upon growth factor and amino acid stimulation, the active Rag complex consisting of RagA/B in the GTP-bound state and RagC/D in the GDP-bound state ($RagA/B^{GTP}$-$RagC/D^{GDP}$) promotes mTORC1 translocation to the lysosome, where GTP-bound Rheb stimulates the mTOR kinase.

As a crucial determinant of small GTPase activity, the guanine nucleotide-loading status of GTPases is regulated by multiple factors including GTPase-activating proteins (GAPs) that stimulate GTP hydrolysis, guanine nucleotide exchange factors (GEFs) that facilitate GDP dissociation, and the guanine nucleotide dissociation inhibitors (GDIs) that prevent GDP dissociation (Cherfils and Zeghouf, 2013). The TSC2 component of the trimeric tuberous sclerosis complex (TSC) is a GAP for Rheb, which inhibits Rheb by converting it from the GTP-bound state to the GDP-bound state (Inoki et al., 2003; Tee et al., 2003). While TSC phosphorylation by the energy sensor AMP-responsive protein kinase (AMPK) promotes its activation, growth factor-induced PI3K/Akt pathway phosphorylates and inactivates TSC (Laplante and Sabatini, 2012). Multiple GAPs have also been identified for Rags including the GATOR1 complex for RagA/B (Bar-Peled et al., 2013), the FLCN-FNIP complex for RagC/D (Tsun et al., 2013), and possibly the leucyl-tRNA synthase for RagD (Han et al., 2012). In addition, the Ragulator complex functions as a GEF for RagA/B, whose activity is induced via the lysosomal v-ATPase upon amino acid stimulation (Bar-Peled et al., 2012). Finally, although GDI proteins are known to control the functions of Rho and Rab subfamilies of small GTPases (Garcia-Mata et al., 2011; Pfeffer and Aivazian, 2004), no GDIs have been described for either Rheb or Rags.

Sestrins are evolutionarily conserved proteins whose expression is upregulated by various environmental insults including genotoxic, oxidative, and nutritional stress (Lee et al., 2013). Whereas mammalian Sestrin1 and Sestrin2 are regulated by p53 (Budanov et al., 2004), Sestrin1 and Sestrin3 as well as the *Drosophila* Sestrin (dSestrin) are target genes of the Foxo family of transcription factors (Chen et al., 2010; Lee et al., 2010; Ouyang et al., 2012). Sestrin overexpression potently suppresses mTORC1 signaling (Budanov and Karin, 2008), and conveys stress signals for the reprogramming of cellular metabolism and the restoration of organismal homeostasis (Lee et al., 2013). Indeed, while dSestrin gain-of-function inhibits mTORC1 signaling and cell growth in *Drosophila*, dSestrin deficiency results in an age-dependent metabolic syndrome caused by mTORC1 hyper-activation (Lee et al., 2010). Likewise, Sestrin2-deficient mice fail to inactivate mTORC1 in the liver during fasting (Bae et al., 2013), and spontaneously elevated mTORC1 signaling is observed in mice devoid of both Sestrin2 and Sestrin3 (Lee et al., 2012). Nevertheless, how Sestrins inhibit mTORC1 remains elusive, although it has been proposed that Sestrins repress mTORC1 signaling through AMPK-dependent activation of TSC2 (Budanov and Karin, 2008; Chen et al., 2010).

In this study, we found that Sestrins can inhibit mTORC1 signaling in the absence of AMPK or TSC2. Surprisingly, when co-expressed with a GTP-bound constitutively active form of RagB ($RagB^{Q99L}$), Sestrins potentiate mTORC1 signaling in the absence of amino acids.

Such opposing activities of Sestrins are in line with the hypothesis that Sestrins function as GDIs for RagA/B by "locking" the wild type (WT) RagA/B or $RagB^{Q99L}$ in GDP- or GTP-bound states respectively. In Sestrins, we mapped an evolutionarily conserved GDI motif that is homologous to that of the Rab GDIs, which is essential for Sestrin inhibition of mTORC1 signaling. A cell-permeable peptide containing the Sestrin GDI motif is able to partially inhibit mTORC1 signaling in a Rag GTPase-dependent manner. Importantly, neonatal mice deficient in all Sestrins have dramatically reduced postnatal survival associated with failed inactivation of mTORC1 signaling in liver, heart, and skeletal muscle during neonatal fasting. Together, these findings reveal Sestrins as a family of GDIs for Rags with essential functions in the control of nutrient sensing and mTORC1 signaling.

RESULTS

TSC2 and AMPK Are Nonessential for Sestrin Inhibition of mTORC1 Signaling

Based on the observation that Sestrins could not repress mTORC1 signaling in TSC2 deficient mouse embryonic fibroblasts (MEFs), previous reports concluded that Sestrin inhibition of mTORC1 signaling was dependent on TSC (Budanov and Karin, 2008; Chen et al., 2010). However, we observed that basal mTORC1 activity in TSC2 deficient MEFs was substantially higher than that in WT MEFs (Figure S1A), raising the possibility that Sestrins might not be expressed at sufficiently high levels in previous studies to inhibit elevated mTORC1 signaling. To test this hypothesis, we overexpressed a FLAG-tagged Sestrin2 at different levels in TCS2 deficient MEFs, and found that Sestrin2, in a dose-dependent manner, inhibited threonine 389 phosphorylation of S6K, a canonical mTORC1 substrate (Figure 1A). In addition, Sestrin2 overexpression corrected the low AKT signaling (Figure 1A) caused by mTORC1-induced feedback inhibition of the PI3K pathway in TCS2 deficient MEFs (Um et al., 2004). To corroborate these findings and investigate whether Sestrins can inhibit mTORC1 signaling via TSC-independent mechanisms in human cells, we overexpressed Sestrin2 in HeLa cells in which TSC2 was knocked down by shRNA. As expected, TSC2 knockdown led to enhanced mTORC1 signaling (Figure S1B). Importantly, Sestrin2 overexpression substantially inhibited mTORC1 activation in TSC2 knockdown cells (Figure S1B). These findings reveal that TSC2 is not essential for Sestrin suppression of mTORC1 activity.

Previous studies have also implicated AMPK in Sestrin inhibition of mTORC1 signaling (Budanov and Karin, 2008; Chen et al., 2010). Although AMPK phosphorylates and activates TSC, it can suppress mTORC1 signaling through TSC-independent pathways such as direct phosphorylation of the mTORC1 component Raptor (Gwinn et al., 2008). To investigate the definitive role of AMPK in Sestrin regulation of mTORC1 signaling, we overexpressed Sestrin2 in MEFs deficient in both AMPKα1 and AMPKα2 ($AMPK^{DKO}$). Unexpectedly, Sestrin2 overexpression repressed mTORC1 signaling in a dose-dependent manner in $AMPK^{DKO}$ MEFs (Figure 1B), in line with reduced cell size (Figure S1C) as a consequence of diminished mTORC1 activity. Taken together, these observations demonstrate that neither TSC2 nor AMPK is essential for Sestrin regulation of mTORC1 signaling.

Sestrins Inhibit Rag-dependent mTORC1 Lysosomal Translocation

The finding that TSC2 was dispensable for Sestrin inhibition of mTORC1 signaling suggested that Sestrins do not interfere with Rheb-dependent mTORC1 activation induced by growth factors. Because amino acid-triggered mTORC1 lysosomal translocation is an obligate step for mTORC1 activation that is independent of the TSC-Rheb axis (Bar-Peled and Sabatini, 2014), we explored the possibility that Sestrins might target the nutrient-sensing pathway for mTORC1 regulation. A previous study showed that amino acid signaling to mTORC1 can be bypassed by expressing a mutant form of the mTORC1 scaffolding subunit Raptor with its C-terminus fused to a 15-amino acid lysosome-targeting signal from Rheb1 (Raptor-Rheb15) (Sancak et al., 2010). Indeed, compared to cells expressing the control GTPase Rap2A, cells expressing Raptor-Rheb15 that tethers mTORC1 to the lysosome were insensitive to Sestrin2-induced mTORC1 suppression (Figure 2A). These observations imply that Sestrins inhibit mTORC1 signaling upstream of mTORC1 lysosomal translocation.

Amino acid-induced mTORC1 translocation is mediated by the Rag subfamily of small GTPases. The active Rag complex, RagA/B^{GTP}-RagC/D^{GDP}, physically interacts with Raptor, and recruits mTORC1 to the lysosome (Sancak et al., 2010). To investigate whether Sestrins regulate mTORC1 via Rag proteins, we used a $RagB^{Q99L}$ mutant that is "locked" in the active GTP-bound state, and renders the Rag complex constitutively active (Sancak et al., 2008). Indeed, when co-expressed with Sestrin2, $RagB^{Q99L}$ but not the control Rap2A fully rescued mTORC1 signaling (Figure 2B).

To directly examine the role of Sestrins in the control of mTORC1 translocation, we established stable cell lines expressing Sestrin1, Sestrin2, or Sestrin3. Cells were starved for 60 minutes in a medium that was depleted of amino acids but contained 10% fetal bovine serum to maintain growth factor signaling, and were re-stimulated with amino acid-replete medium for 15 minutes. As expected, mTORC1 was dispersed in the cytosol in the absence of amino acids, and amino acid re-stimulation triggered mTORC1 translocation to the lysosome marked by Lamp2 staining in control HeLa cells (Figure 2C). In contrast, amino acid-induced mTORC1 translocation was markedly inhibited in cells expressing either of the Sestrins (Figure 2C), while the lysosomal localization of Rag molecules was not affected (Figure S2). These observations suggest that Sestrins repress mTORC1 signaling through the inhibition of amino acid-induced Rag activation and mTORC1 lysosomal translocation.

Sestrins Directly Interact with Rags

To investigate whether Sestrins regulate Rag activation through protein-protein interactions, we co-transfected HA-GST-tagged Rag heterodimers or control Rap2A with FLAG-tagged Sestrin2, and evaluated their interactions by co-immunoprecipitation. We found that Rag complexes with different combinations of GTP- and GDP-loading states but not the control Rap2A co-immunoprecipitated with Sestrin2 (Figure 3A). Similar observations were made with FLAG-tagged Sestrin1 and Sestrin3 (data not shown). To interrogate these interactions more stringently, we established HeLa cell lines expressing FLAG-tagged Sestrins or RFP, and evaluated their association with endogenous Rag proteins. We found that Rags could co-immunoprecipitate with all Sestrins, but not RFP (Figure 3B), although the expression level

of RFP was much higher than those of Sestrins (Figure 3B). The association between Sestrins and Rags was further corroborated by the observation that a Flag-tagged Sestrin was partially co-localized with RagC in cells (Figure S3A). To test whether Sestrins directly interact with the Rag complex, we expressed and purified GST-tagged Sestrin2 (Figure S3B) and His-tagged RagA and RagC heterodimer (Figure S3C) from bacteria. In an *in vitro* GST pull-down assay, we found that the His-RagA-RagC dimer physically interacts with GST-Sestrin2, but not GST alone (Figure 3C). Taken together, these observations demonstrate that Sestrins can interact with the Rag complex *in vivo* and *in vitro*.

Sestrins Enhance RagB^{Q99L}-induced mTORC1 Activation under the Amino Acid Starvation Condition

The finding that Sestrins physically interact with the Rag complex implies that Sestrins may directly modulate Rag GTPase activation. We showed that under the condition of amino acid starvation followed by amino acid re-stimulation, RagB^{Q99L} could fully rescue the mTORC1 suppression induced by Sestrins (Figure 2B). We also observed that the GTP-"locked" RagB^{Q99L} mutant induced mTORC1 signaling in the absence of amino acids (Figure 4A), as expected. Surprisingly, co-expression of Sestrin1, Sestrin2, or Sestrin3 with RagB^{Q99L} substantially enhanced mTORC1 signaling (Figure 4A and data not shown), which was not observed when Sestrins were co-expressed with Raptor-Rheb15 (Figure S4). These observations reveal that Sestrins can either inhibit or potentiate mTORC1 signaling; and such opposing activities function upstream of mTORC1 lysosomal translocation, and are dependent on the guanine nucleotide loading status of the Rag GTPases.

The RagB^{Q99L} mutant was generated based on the finding that the corresponding glutamine 61 in Ras GTPase is required for the nucleophilic attack of the γ-phosphate of GTP to trigger its hydrolysis to GDP (Boguski and McCormick, 1993). When this glutamine is mutated to other amino acid residues, Ras-bound GTP cannot be hydrolyzed and the GTPases are "locked" into the GTP-bound state. However, detailed analysis of the biochemical properties of Ras^{Q61L} showed that this mutant has a six-fold faster GTP dissociation rate compared to that of wild-type Ras due to the inability of the hydrophobic leucine to stabilize GTP (Krengel et al., 1990). Based on these findings, we hypothesized that Sestrins might enhance RagB^{Q99L}-induced mTORC1 activation by preventing GTP dissociation from RagB^{Q99L}.

To test this hypothesis, we generated another RagB mutant (RagB^{Q99H}) by replacing glutamine 99 to histidine, which is analogous to the glutamine 61 to histidine mutant of Ras (Ras^{Q61H}). A previous study showed that similar to Ras^{Q61L}, Ras^{Q61H} cannot hydrolyze GTP, but unlike Ras^{Q61L}, Ras^{Q61H} has a normal GTP dissociation rate that is comparable to that of wild-type Ras (Krengel et al., 1990). To investigate the mTORC1-stimulating functions of RagB^{Q99H} and RagB^{Q99L}, we established RagB^{Q99H}-, RagB^{Q99L}- or control Rap2A-expressing cell lines that co-expressed Sestrin2 or RFP. RagB^{Q99H}-expressing cells had stronger mTORC1 signaling than that of RagB^{Q99L}-expressing cells (Figure 4B and 4C), which was in line with the predicted increase in stability of GTP binding to RagB^{Q99H}. Interestingly, Sestrin2 had a lesser impact on RagB^{Q99H}-induced mTORC1 activation than that triggered by RagB^{Q99L} (Figure 4B and 4C). To directly test whether Sestrin2 could

stabilize $RagB^{Q99L}$ GTP binding, we examined the guanine nucleotide loading status of $RagB^{Q99L}$ in RFP- or Sestrin2-expressing cells that had been pulse labeled with ^{32}P orthophosphate. A HA-GST-tagged $RagB^{Q99L}$ was pulled-down with glutathione beads, and the bound nucleotides were eluted and separated by thin-layer chromatography (TLC). We found that Sestrin2 increased GTP binding to $RagB^{Q99L}$ by more than 2 fold (Figure 4D). Taken together, these observations demonstrate that Sestrins have the unexpected function of enhancing mTORC1 signaling induced by a GTPase-deficient RagB mutant, which is associated with the stabilization of GTP binding to $RagB^{Q99L}$.

Sestrins Are GDIs for RagA and RagB

To investigate whether Sestrins regulated guanine nucleotide loading of endogenous Rags, we immunoprecipitated RagA and analyzed RagA-bound nucleotides by TLC. In line with a previous study (Sancak et al., 2008), amino acid stimulation triggered a GDP to GTP exchange resulting in a higher proportion of GTP-loaded RagA than observed in amino acid-starved cells (Figure 5A). This amino acid-induced nucleotide exchange was completely inhibited in Sestrin2-expressing cells (Figure 5A). These observations suggest that Sestrin-mediated suppression of mTORC1 signaling results from its inhibition of RagA GDP dissociation upon amino acid stimulation.

The ability of Sestrin2 to stabilize RagA GDP binding is most consistent with its role as a GDI. To test this hypothesis, we adopted a previously described GEF/GDI assay (Bar-Peled et al., 2012) in which the conserved aspartic acid in the “NKxD” motif of RagB and RagC was mutated to asparagine ($RagB^{D163N}$ and $RagC^{D181N}$, designated as $RagB^{X}$ and $RagC^{X}$ respectively). This mutation changes the base specificity of GTPases from guanine to xanthosine (Hoffenberg et al., 1995; Schmidt et al., 1996). We could thus pre-load the recombinant RagA/B-$RagC^{X}$ or $RagB^{X}$-RagC/D (Figure S5A) with GDP and XTP, which would result in GDP binding to WT Rags and XTP binding to $RagB^{X}$ or $RagC^{X}$. ^{35}S-labeled GTP was subsequently added to initiate nucleotide exchange. We found that Sestrin2 inhibited GDP to GTP exchange in RagA/B, but not in RagC/D (Figure 5B). Furthermore, Sestrin2 did not stimulate the hydrolysis of Rag-bound GTP (Figure S5B), and therefore was not a GAP for Rags. These findings demonstrate that Sestrins are GDIs for RagA and RagB.

A GDI Motif Is Necessary and Sufficient for Sestrin Inhibition of mTORC1 Signaling

To gain molecular insights into the GDI activity of Sestrins, we aligned the sequences of Sestrins to those of RhoGDI and Rab GDI. Although Sestrins did not share overall sequence similarities with RhoGDI or Rab GDI (data not shown), a peptide motif near the C-termini of all Sestrins from fly, mouse, and human was highly homologous to the Rab GTPase-binding motif of Rab GDI (Schalk et al., 1996) (Figure 6A).

Structural analysis of Rab GDI has revealed that the arginine residue within the motif forms a hydrogen bond with the aspartic acid residue from the switch 2 region of Rab, which stabilizes the coordination of Mg^{2+} that is essential for guanine nucleotide binding (Rak et al., 2003). To investigate whether the analogous arginine residue in Sestrins was essential for Sestrin regulation of mTORC1 signaling, we mutated arginine 419 in Sestrin2 to alanine, and established a stable cell line expressing this mutant ($Sestrin2^{419A}$). Indeed, compared to

WT Sestrin2, Sestrin2^{419A} was unable to inhibit mTORC1 signaling in cells cultured with amino acid-replete medium (Figure 6B, lanes 1, 5, and 13).

To determine whether Sestrin2^{419A} regulation of mTORC1 activation might be dependent on the strength of nutrient signaling, we cultured cells in medium with different concentrations of amino acids. Lowering the concentration of amino acids in control cells led to a gradual decline of mTORC1 signaling (Figure 6B, lanes 1 – 4), which was exacerbated in cells expressing Sestrin2 (Figure 6B, lanes 5 – 8). Surprisingly, the effect of amino acid dosage on mTORC1 signaling was much attenuated in cells expressing Sestrin2^{419A}, with 5% amino acids maintaining a high level of mTORC1 activity (Figure 6B, lanes 13 – 15). Residual mTORC1 activity could still be detected in medium depleted of amino acids (Figure 6B, lane 16), suggesting that Sestrin2^{419A} might function as a dominant negative mutant. Indeed, further characterization of the Sestrin2^{419A} mutant showed that Sestrin2 binding to Rags was not compromised with the arginine 419 to alanine substitution (Figure S6 and data not shown).

Because the dominant negative effect of Sestrin2^{419A} was partial, we reasoned that positively charged residues other than the arginine might compensate for the GDI activity of Sestrins. A closer examination of the sequence alignment revealed two highly conserved lysines in all Sestrins (K422 and K426 in Sestrin2), which are not present in Rab GDI (Figure 6A). Replacement of these two lysines either alone or in combination with alanines did not have a major effect on Sestrin2 inhibition of mTORC1 activation (Figure 6B, lanes 17 – 24, and data not shown). However, a Sestrin2 mutant with the replacement of both lysines and arginine to alanines (Sestrin2^{AAA}) rendered the cells almost completely insensitive to amino acid starvation-triggered mTORC1 inactivation (Figure 6B, lane 9 – 12). In line with its inability to inhibit mTORC1 signaling, Sestrin2^{AAA} was mostly devoid of its GDI activity towards RagB (Figure 6C). Furthermore, cells expressing Sestrin2^{AAA} were refractory to amino acid starvation-triggered mTORC1 lysosome dissociation (Figure 6D). These findings have thus identified a functional GDI motif in Sestrins, and supported the conclusion that Sestrins target the Rag-dependent nutrient-sensing pathway to control mTORC1 signaling.

To investigate whether the GDI motif of Sestrins might be sufficient to inhibit mTORC1 signaling, we generated a cell-permeable peptide, Tat-Sestrin2-GDI motif (T-G), composed of the HIV-1 Tat protein transduction domain (Van den Berg and Dowdy, 2011) attached via a di-glycine linker to a 22-amino acid peptide encompassing the GDI motif of Sestrin2 (amino acids 410 – 431) (Figure 6E). In addition, the GDI motif was randomly shuffled to generate a control Tat-Sestrin2-GDI motif-Scramble peptide (T-S) (Figure 6E). Treatment of HEK293T cells with T-G, but not T-S, resulted in a dose-dependent repression of mTORC1 signaling (Figure 6F). Importantly, T-G failed to inhibit mTORC1 activation in HEK293T cells stably expressing RagB^{Q99L} that “locks” RagB in the GTP-bound state, and renders cells insensitive to amino acid deprivation-triggered mTORC1 inactivation (Figure 6F). These findings demonstrate that the GDI motif of Sestrins by itself is able to target the Rag GTPases to suppress mTORC1 signaling.

Loss of Sestrins Renders mTORC1 Signaling Insensitive to Nutrient Status *In Vivo*

The studies described thus far were carried out with overexpression of WT or mutant Sestrins. To investigate whether endogenous Sestrins were essential regulators of Rag-dependent mTORC1 signaling, we wished to generate Sestrin deficient mice. Overexpression of Sestrin1, Sestrin2, or Sestrin3 inhibited mTORC1 activation (Figure 2C), raising the possibility that they might be functionally redundant. To investigate the total contribution of Sestrins, we generated null alleles for *Sesn1*, *Sesn2*, and *Sesn3* (Figure S7A – S7D). Mice harboring these alleles were crossed with each other to generate the triple-knockout (TKO) mice. We found that mice with the deletion of 5 of the 6 Sestrin alleles were born with the expected Mendelian ratios (data not shown). However, Sestrin TKO pups were dramatically underrepresented when genotyped at 10 days after birth (Figure 7A).

We suspected that deletion of all Sestrin alleles in mice might result in an embryonic lethal phenotype. Surprisingly, when neonatal mice were genotyped right after birth, Sestrin TKO mice were born at an expected Mendelian ratio, and appeared indistinguishable from their wild-type littermates (Figure 7A and data not shown). These findings suggest that Sestrins are not required for embryonic development, but are important for postnatal survival. Intriguingly, a similar neonatal lethal phenotype was observed in $RagA^{GTP/GTP}$ knockin mice that could not terminate mTORC1 signaling during the fasting period that occurs in mammals between birth and suckling (Efeyan et al., 2013). Indeed, we found that mTORC1 signaling was constitutively active in the liver, heart and skeletal muscle (limbs) of TKO mice during neonatal fasting (Figure 7B). In particular, mTORC1 activity in the liver and limbs was inversely associated with the Sestrin gene dosage, which was in line with the finding that all Sestrins were expressed in these tissues (Figures 7B and S7E, and data not shown). In contrast, Sestrin1, but not Sestrin2 or Sestrin3, was expressed at high levels in the heart (Figure 7B and data not shown), and the loss of Sestrin1 alone was sufficient to render mTORC1 signaling resistant to neonatal fasting in this organ (Figures 7B and S7F). Taken together, these findings demonstrate that Sestrins have family member-specific as well as redundant functions in repressing mTORC1 signaling in different tissues during neonatal fasting, and the loss of all three Sestrins triggers a neonatal lethal phenotype.

To further investigate Sestrin control of mTORC1 signaling, we used MEFs that expressed all three Sestrins (Figure S7G - S7I). We derived MEFs from wild-type, $Sesn1^{+/-}2^{-/-}3^{+/-}$, $Sesn1^{+/-}2^{-/-}3^{-/-}$, and $Sesn1^{-/-}2^{-/-}3^{-/-}$ embryos, and found that the sensitivity of mTORC1 signaling to amino acids was dependent on Sestrin gene dosage: the fewer alleles of Sestrins, the less dependence of MEFs on amino acids (Figure S7J). Importantly, whereas mTORC1 signaling in wild-type MEFs was completely inhibited within 30 minutes of amino acid starvation, $Sesn1^{-/-}2^{-/-}3^{-/-}$ MEFs maintained mTORC1 activation after prolonged amino acid deprivation (Figure 7C). As another major nutrient, glucose also regulates mTORC1 activity partially through Rag GTPases (Efeyan et al., 2013). We found that $Sesn1^{-/-}2^{-/-}3^{-/-}$ MEFs were also resistant to mTORC1 inactivation triggered by glucose, or glucose and amino acid deprivation (Figure 7D). In line with these observations, substantial amounts of mTORC1 were localized on the lysosome in $Sesn1^{-/-}2^{-/-}3^{-/-}$ MEFs under the condition of amino acid starvation (Figure 7E). In contrast, mTORC1 signaling in $Sesn1^{-/-}2^{-/-}3^{-/-}$ MEFs was still dependent on growth factors (Figure 7D). In addition,

unlike *Tsc2*$^{-/-}$ MEFs that had increased mTORC1 signaling when cultured in complete medium, *Sesn1*$^{-/-}$*2*$^{-/-}$*3*$^{-/-}$ MEFs did not show enhanced mTORC1 activation under the same conditions (Figure S7K), suggesting that the maximum capacity of mTORC1 signaling was not increased in the absence of Sestrins. In summary, these data demonstrate that Sestrins are essential regulators of Rag GTPases, with Sestrin1, Sestrin2, and Sestrin3 functioning redundantly to suppress nutrient- but not growth factor-induced mTORC1 signaling (Figure S7L).

DISCUSSION

The landmark discovery of Rag proteins as central regulators of amino acid signaling to mTORC1 has opened up new avenues for the study of nutrient-sensing pathways (Kim et al., 2008; Sancak et al., 2008). As GTPases, Rags were predicted to be regulated by GEFs and GAPs. Indeed, recent studies have identified a GEF (Bar-Peled et al., 2012) and several GAPs for Rags (Bar-Peled et al., 2013; Han et al., 2012; Tsun et al., 2013). Here we show that Rag proteins are also subject to regulation by the Sestrin family of GDIs.

Our study began by revising the role of TSC2 in Sestrin control of mTORC1 signaling. Using cells expressing different amounts of Sestrin2, we found that TSC2 was partially required, but not essential for Sestrin2 inhibition of mTORC1 activation. Although TSC has established functions in the control of stress and growth factor-dependent mTORC1 signaling, its role in amino acid signaling to mTORC1 remains incompletely understood. The observation that amino acid deprivation can reduce but not fully inactivate mTORC1 signaling in TSC2 deficient MEFs implies TSC-dependent and -independent mechanisms of mTORC1 inactivation. A recent study showed that during amino acid starvation, the inactive Rag complex recruits TSC to the lysosome to inactivate Rheb (Demetriades et al., 2014). As GDIs for Rags, Sestrins may maintain the lysosomal localization of TSC by “locking” RagA/B in the GDP-bound state, which may explain why Sestrin inhibition of mTORC1 signaling is partially TSC2-dependent.

The name “GDI” was coined by the discovery of RhoGDI as an inhibitor of GDP dissociation from Rho GTPase (Fukumoto et al., 1990). Later studies have revealed that RhoGDI and Rab GDI recognize the geranylgeranylated Rho and Rab proteins, extract these GTPases from endomembranes, and maintain them as soluble inactive complexes in the cytosol (Cherfils and Zeghouf, 2013). Sestrins display classical GDI activity towards Rag GTPases, but do not affect their lysosomal localization, making them a unique subfamily of GDIs. In fact, Rag GTPases do not have a membrane targeting sequence, but are anchored to the lysosomal surface by the Ragulator complex that also functions as a GEF for RagA/B (Bar-Peled et al., 2012; Sancak et al., 2010). Together, these findings suggest that compared to the other subfamily of small GTPases, Rags are regulated via distinct mechanisms of endomembrane targeting and activation.

Both GEF induction of GDP displacement and GAP stimulation of GTP hydrolysis consist of multistep reactions. Indeed, these GTPase regulators are typically large proteins and in many cases protein complexes (Cherfils and Zeghouf, 2013), which include the five protein complex GEF (Ragulator) and the three protein complex GAP (GATOR1) for RagA/B (Bar-

Peled et al., 2012; Bar-Peled et al., 2013). Furthermore, GEFs and GAPs are present in large numbers, and can sometimes outnumber their target GTPases by more than 3 to 1 as in the case of Rho GTPases (Cote and Vuori, 2007; Rossman et al., 2005). In contrast, all GDIs identified so far are single chain polypeptides with only a few family members (Cherfils and Zeghouf, 2013). For example, Rab (65 members) and Rho (22 members) GTPases each have only three GDIs (Rojas et al., 2012). Therefore, it is rather unusual that there are three Sestrin molecules functioning as GDIs for Rags in mammals. Such an expansion of functional GDI members suggests that Sestrins were selected during evolution as important regulators of Rag GTPases.

Because of the relatively simple architecture of GDI molecules, their activity can be ascribed to specific motifs. We have identified a GDI motif in Sestrins that is similar to that of Rab GDI. Although the ultimate demonstration of GDI mechanism awaits the co-crystal structure of a Rag dimer in complex with Sestrin, we can deduce from our biochemical data that the conserved arginine (R419 in Sestrin2) is largely responsible for Sestrin stabilization of guanine nucleotides in RagA/B. The $Sesn2^{419A}$ mutant we generated functions as a partially dominant negative mutant. Interestingly, a recent report has identified a Sestrin2 R419W mutation in colorectal adenocarcinoma (Cancer Genome Atlas, 2012). Future studies will determine whether this mutation creates a Sestrin2 dominant negative mutant to render tumor cells resistant to nutrient starvation-triggered mTORC1 inactivation.

Why Sestrins function as GDIs for RagA/B, but not RagC/D, is presently unknown. It is plausible that within the Rag heterodimer, Sestrins may directly bind to RagA or RagB, but not RagC or RagD. In addition, the amino acid sequence differences in the switch 2 regions between RagA/B and RagC/D may explain why the GEF, GAPs, and GDIs are specific to either RagA/B or RagC/D. The switch 2 motifs of RagA/B have the sequence of “Leu-Trp-Asp-Cys-Gly-Gly-Gln-Asp” that ends with a conserved negatively charged aspartic acid. In contrast, the switch 2 motifs of RagC/D are “Ile-Trp-Asp-Phe-Pro-Gly-Gln-Met/Ile” ending with the hydrophobic methionine or isoleucine. It is therefore possible that the negatively charged aspartic acid in switch 2 of RagA/B interacts with the positively charged amino acids within the GDI motif of Sestrins.

An interesting observation made from this study is that Sestrins promote mTORC1 activation induced by the RagB mutant $RagB^{Q99L}$. Although counterintuitive at first glance, this phenomenon precisely reflects the GDI activity of Sestrins towards Rags. Because $RagB^{Q99L}$ is devoid of GTPase activity, $RagB^{Q99L}$ induction of mTORC1 signaling is modulated by its guanine nucleotide binding capacity. In fact, it is the rapid GTP dissociation rate that makes $RagB^{Q99L}$ subject to regulation by Sestrin2, because Sestrin2 has minimal effect on $RagB^{Q99H}$, which has a normal GTP dissociation rate. Therefore, for endogenous wild-type RagA/B proteins, Sestrins may preferentially stabilize their GDP- rather than GTP-bound states, which is in line with the observation that Sestrin overexpression preferentially blocks the release of GDP from RagA without affecting much of GTP binding.

The identification of Sestrins as GDIs for Rag GTPases may help to elucidate the precise biochemical mechanisms of nutrient sensing. The current model of amino acid regulation of

mTORC1 suggests that amino acids control the localization of mTORC1 by modulating the guanine nucleotide loading status of Rags (Bar-Peled and Sabatini, 2014; Jewell et al., 2013). As a GEF for RagA/B, the Ragulator complex may sense the conformational change of v-ATPase induced by amino acids present within the lysosome lumen (Bar-Peled et al., 2012). Because Sestrin overexpression potently suppresses mTORC1 signaling, its GDI activity must be regulated as well. It is possible that amino acid-induced signaling promotes the dissociation of Sestrins from Rags through an unknown GDF (GDI Displacement Factor). It has been shown that GEFs themselves can function as GDFs for the same GTPase (Schoebel et al., 2009). It will therefore be interesting to investigate whether the Ragulator complex can displace Sestrins from RagA/B upon amino acid stimulation.

Most components of the Ragulator and GATOR1 complexes are newly identified proteins with few studies on how their expression is regulated. In contrast, as target genes of the p53 and Foxo families of transcription factors, Sestrins are not only constitutively expressed but are also regulated by genotoxic, oxidative, and nutritional stress (Lee et al., 2013). A recent study showed that Sestrin2 expression is induced by the transcription factor ATF4 in response to ER stress signals (Bruning et al., 2013). Furthermore, oncogenic AKT signaling pathway downstream of growth factors is aberrantly activated in most tumors, and may activate mTORC1 signaling not only through the AKT-TSC pathway but also via the AKT-Foxo-Sestrin axis. Interestingly, concomitant with the evolution of growth factor signaling pathways, Sestrin orthologs are present in metazoans but not in protozoans such as yeast (Budanov et al., 2010), although Rag-dependent nutrient sensing is well conserved in all eukaryotes. These observations suggest that Sestrins are metazoan specie-specific GDIs for Rag GTPases, and reside in a critical node of regulation by coupling cellular stress and growth signals to mTORC1 activation.

The postnatal survival defect and the constitutive mTORC1 signaling during neonatal fasting in mice devoid of all Sestrin molecules are similar to those of the recently generated *RagA*$^{GTP/GTP}$ mice (Efeyan et al., 2013). These observations support the conclusion that Sestrins modulate the guanine nucleotide loading status of RagA/B *in vivo*. Nevertheless, it remains to be determined whether mTORC1 inhibitors such as rapamycin can correct the neonatal lethal phenotype in Sestrin deficient mice.

Dysregulated mTORC1 signaling has been associated with a plethora of pathological conditions including cancer, diabetes, and aging. Indeed, the well-characterized mTOR inhibitor rapamycin shows an anti-aging effect from yeast to mammals (Fontana et al., 2010). However, significant side effects have been observed after prolonged treatment, which prevents its use in humans as an anti-aging drug (Lamming et al., 2013). It is believed that some of the side effects of long-term rapamycin treatment are caused by its inhibition of mTORC2 (Lamming et al., 2012). Therefore, mTORC1 specific inhibitors are actively sought in the field. In this study, we have identified a cell-permeable peptide containing the Sestrin GDI motif capable of suppressing mTORC1 signaling in a Rag GTPase dependent manner in cultured cells. Although the inhibitory effect of this peptide on mTORC1 is weaker than that of rapamycin, our proof-of-concept study heralds the future development of mTORC1 specific inhibitors through targeting the nutrient-sensing Rag GTPases.

EXPERIMENTAL PROCEDURES

For detailed protocols, see Extended Experimental Procedures.

Amino Acid, Glucose, and Serum Starvation and Re-stimulation

HEK293T, HeLa, and MEFs were treated with doxycycline to induce Sestrin expression. Cells were rinsed once with amino acid- and/or glucose-free DMEM, incubated with amino acid- and/or glucose-free DMEM supplemented with 10% dialyzed FBS for 1 h, and stimulated with amino acid and glucose-replete DMEM for 10 – 20 min. For serum starvation, cells were incubated with DMEM without FBS for 2 h. After treatment, cells were lysed, and protein extracts were prepared for immunoblotting.

Immunofluorescence

Cells were plated on Coverslips. Twenty-four hours later, cells were either amino acid-starved, or starved followed by amino acid re-stimulation. Cells were rinsed with PBS and fixed with 4% paraformaldehyde. The slides were rinsed with PBS, and cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After rinsing with PBS, the slides were incubated with primary antibodies in 5% normal donkey serum for 1 – 3 h, rinsed with PBS, and incubated with secondary antibodies for 1 h. Slides were washed with PBS, mounted on glass.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Sestrins prevent amino acid-induced mTORC1 recruitment to the lysosome
- Sestrins are guanine nucleotide dissociation inhibitors for RagA and RagB
- A cell-permeable peptide containing the GDI motif inhibits mTORC1 activation
- Sestrin deficiency renders mTORC1 signaling insensitive to nutrient status *in vivo*

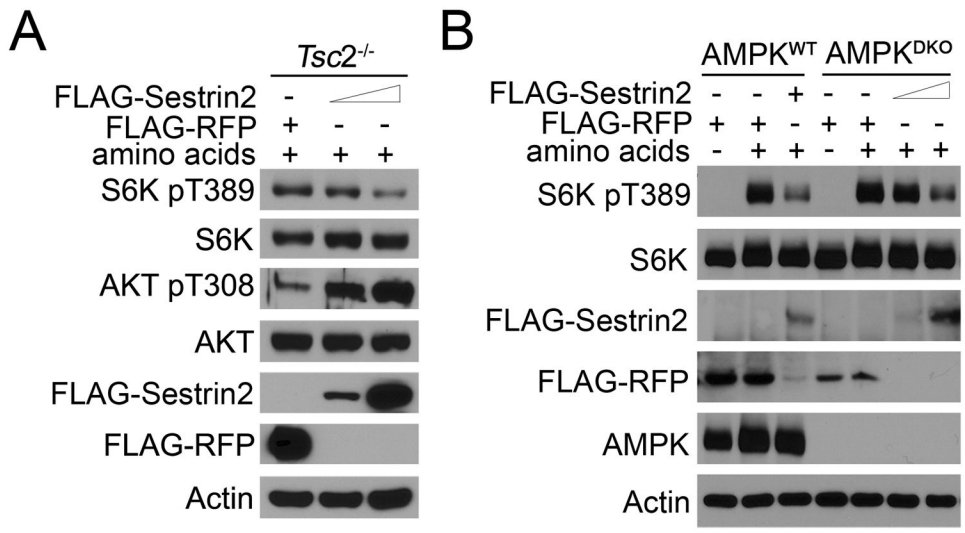

Figure 1. Sestrin2 Inhibits mTORC1 Signaling in the Absence of TSC2 or AMPK

(A) TSC2-deficient MEFs stably expressing FLAG-tagged Sestrin2 or RFP were cultured in complete medium. Total cell lysates were analyzed by immunoblotting.

(B) Wild-type (WT) or AMPK^{DKO} MEFs stably expressing FLAG-tagged Sestrin2 or RFP were cultured in complete medium or starved for amino acids for 60 min, and analyzed as in **(A)**.

See also Figure S1.

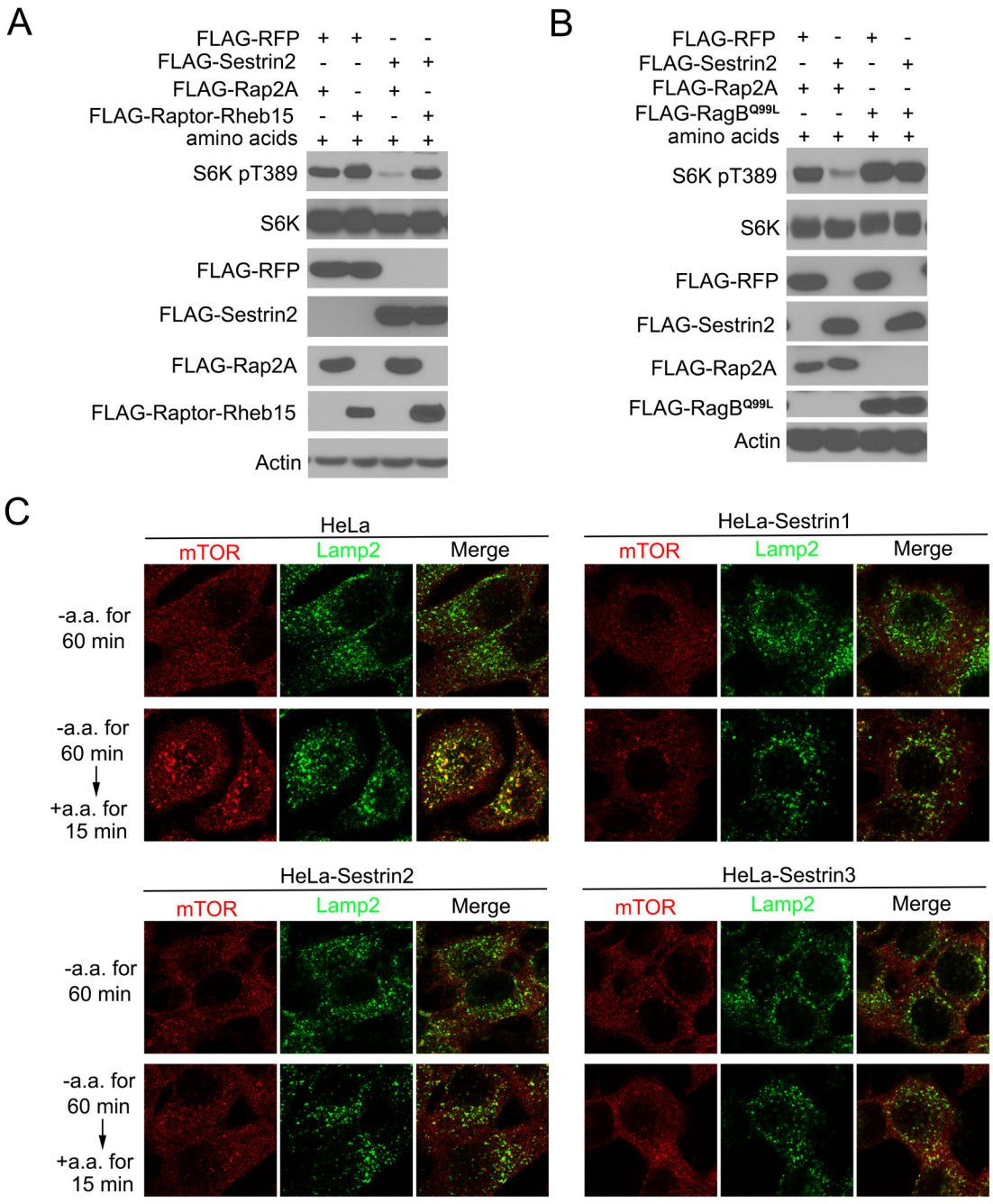

Figure 2. Sestrins Function Upstream of the Rag GTPases to Inhibit Amino Acid-Induced mTORC1 Lysosomal Translocation and Signaling

(A) HEK293T cells stably expressing indicated proteins were starved for amino acids for 60 min, and re-stimulated with amino acids for 10 min. Total cell lysates were analyzed by immunoblotting.

(B) HEK293T cells stably expressing indicated proteins were treated and analyzed as in **(A)**.

(C) HeLa cells or HeLa cells stably expressing indicated proteins were starved for amino acids for 60 min, and were either left untreated or re-stimulated with amino acids for 15 min. The localization of mTOR and Lamp2 was determined by immunostaining.

See also Figure S2.

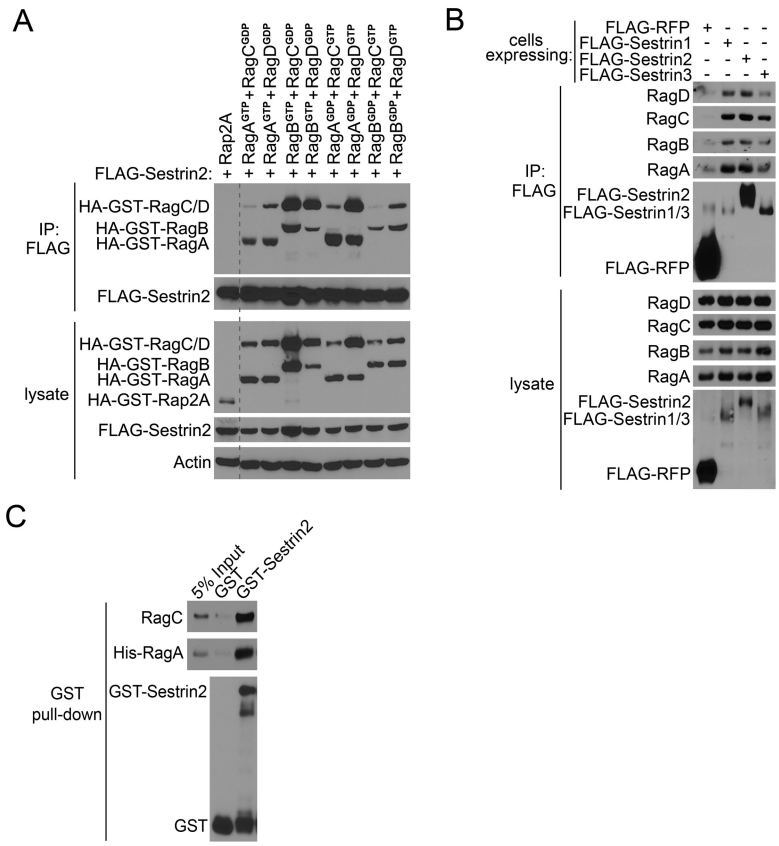

Figure 3. Sestrins Directly Interact with the Rag GTPases

(A) HEK293T cells were co-transfected with constructs encoding indicated proteins. Total cell lysate or the anti-FLAG immunoprecipitate (IP) was analyzed by immunoblotting. All lanes were from the same blot, and irrelevant lanes were removed and indicated by a dashed line.

(B) HeLa cells stably expressing indicated proteins were subject to crosslinking with DSP prior to cell lysis. Total cell lysate or the anti-FLAG immunoprecipitates was analyzed by immunoblotting.

(C) GST, GST-Sestrin2, and His-RagA-RagC dimer were purified from bacteria and used in the GST pull-down assay. The amounts of RagA, RagC, GST, and GST-Sestrin2 proteins were determined by immunoblotting.

See also Figure S3.

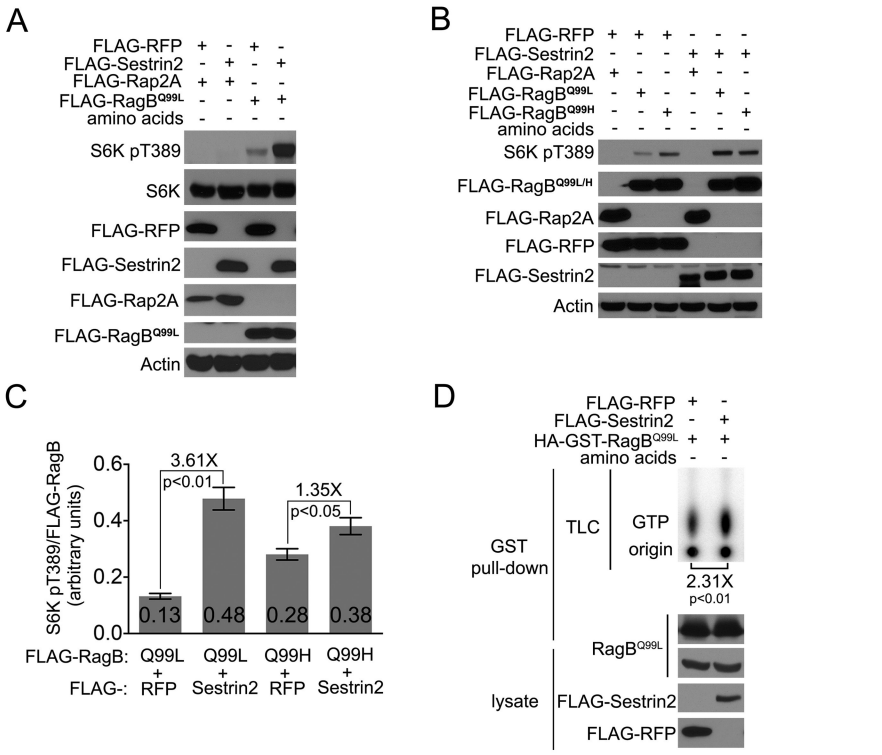

Figure 4. Sestrin2 Stabilizes GTP Binding to RagB^{Q99L} and Potentiates RagB^{Q99L}-Induced mTORC1 Signaling

(A) HEK293T cells stably expressing indicated proteins were starved for amino acids for 60 min. Total cell lysates were analyzed by immunoblotting.

(B–C) HEK293T cells stably expressing indicated proteins starved for amino acids for 60 min. Total cell lysates were analyzed by immunoblotting **(B)**. The effect of Sestrin2 on RagB^{Q99L}- or RagB^{Q99H}-induced S6K phosphorylation was normalized to the expression level of RagB by the Image J software

(C). The arbitrary units represent the normalized mean ± SEM for n = 3. The relative fold change between RFP-expressing and Sestrin2-expressing cells, and the p values between the measurements are shown.

(D) HEK293T cells stably expressing indicated proteins were transfected with constructs encoding the HA-GST-RagB^{Q99L} and FLAG-RagC^{D181N} dimer. The RagB^{Q99L}-bound guanine nucleotides were analyzed by TLC.

See also Figure S4.

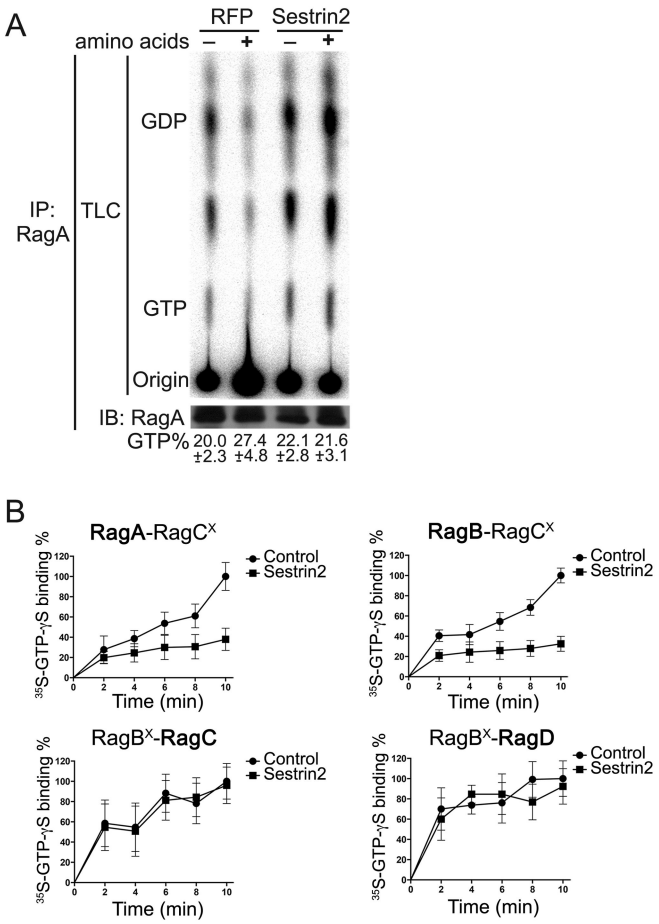

Figure 5. Sestrin2 Is a GDI for RagA/B

(A) HEK293T cells stably expressing indicated proteins were labeled with ^{32}P orthophosphate for 4 h, starved for amino acids for 50 min and restimualted with amino acids for 10 min, and RagA was immunoprecipitated with anti-RagA. The RagA-bound guanine nucleotides were eluted and separated by TLC. The percentage of GTP-bound RagA was quantified, and is shown as the normalized mean ± SEM for n = 3.

(B) The different combinations of Rag dimers were pre-loaded with GDP and XTP, and were incubated with ^{35}S-GTPγS in the absence or presence of Sestrin2. Aliquots of the samples were taken at 2, 4, 6, 8, and 10 min, and the amounts of exchanged ^{35}S-GTPγS were determined in a filter-binding assay. The relative fractions of the exchanged guanine nucleotides were normalized, and are presented as mean ± SEM for n = 3.

See also Figure S5.

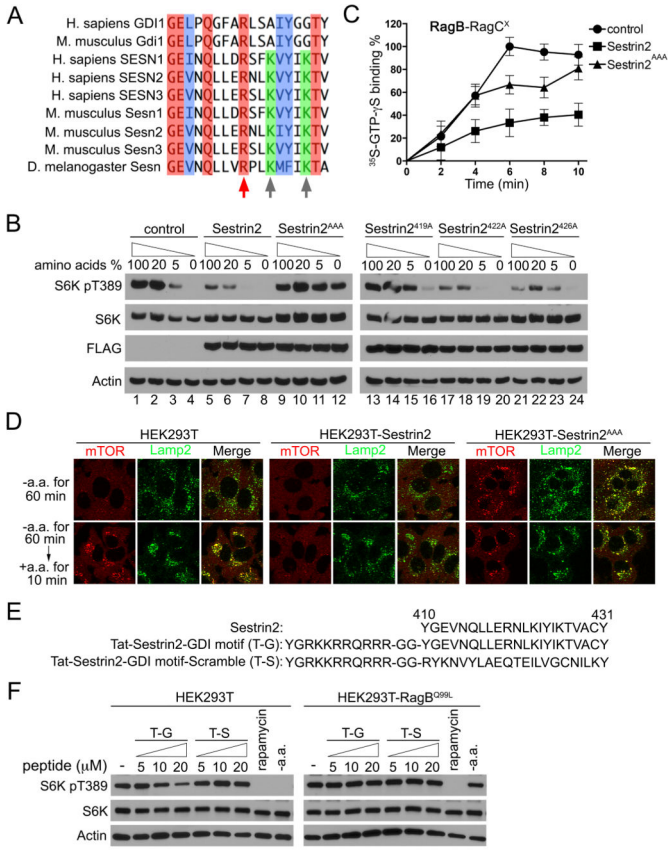

Figure 6. The GDI Activity of Sestrin Is Necessary and Sufficient for Inhibiting mTORC1 Activation

(A) Sequence alignment of a putative GDI motif from Sestrins to that of RabGDI is shown. The conserved amino acids are labeled in red and blue, and the two positively charged lysines present specifically in Sestrins are labeled in green. Arrows indicate the amino acid residues that are mutated to alanines.

(B) HEK293T cells stably expressing indicated proteins were cultured in medium with different concentrations of amino acids for 60 min. Total cell lysates were analyzed by immunoblotting.

(C) The RagB-RagC^{X} dimer was pre-loaded with GDP and XTP, and was incubated with ^{35}S-GTPγS in the absence or presence of Sestrin2 or Sestrin2^{AAA}. Aliquots of the samples were taken at 2, 4, 6, 8, and 10 min, and the amounts of exchanged ^{35}S-GTPγS were determined in a filter-binding assay. The relative fractions of the exchanged guanine nucleotides were normalized, and are presented as mean ± SEM for n = 3.

(D) HEK293T cells stably expressing indicated proteins were starved for amino acids for 60 min, and were either left untreated or re-stimulated with amino acids for 10 min. The localization of mTOR and Lamp2 was determined by immunostaining.

(E) Sequences of mouse Sestrin2 amino acids 410 – 431, Tat–Sestrin2-GDI motif (T-G) and Tat- Tat–Sestrin2-GDI motif-Scramble (T-S) control peptides.

(F) HEK293T or HEK293T stably expressing FLAG-RagB^{Q99L} were incubated with DMEM containing the indicated concentrations of peptides for 60 min. Cells were also

deprived of amino acids or incubated with rapamycin (100 ng/ml) for 60 min as controls. Total cell lysates were analyzed by immunoblotting.
See also Figure S6.

A

Genotyping on day 10

# of pups	# of TKO	TKO(%) observed	TKO(%) expected
59	2	3.4%	25.0%

Genotyping at birth

# of pups	# of TKO	TKO(%) observed	TKO(%) expected
85	20	23.5%	25.0%

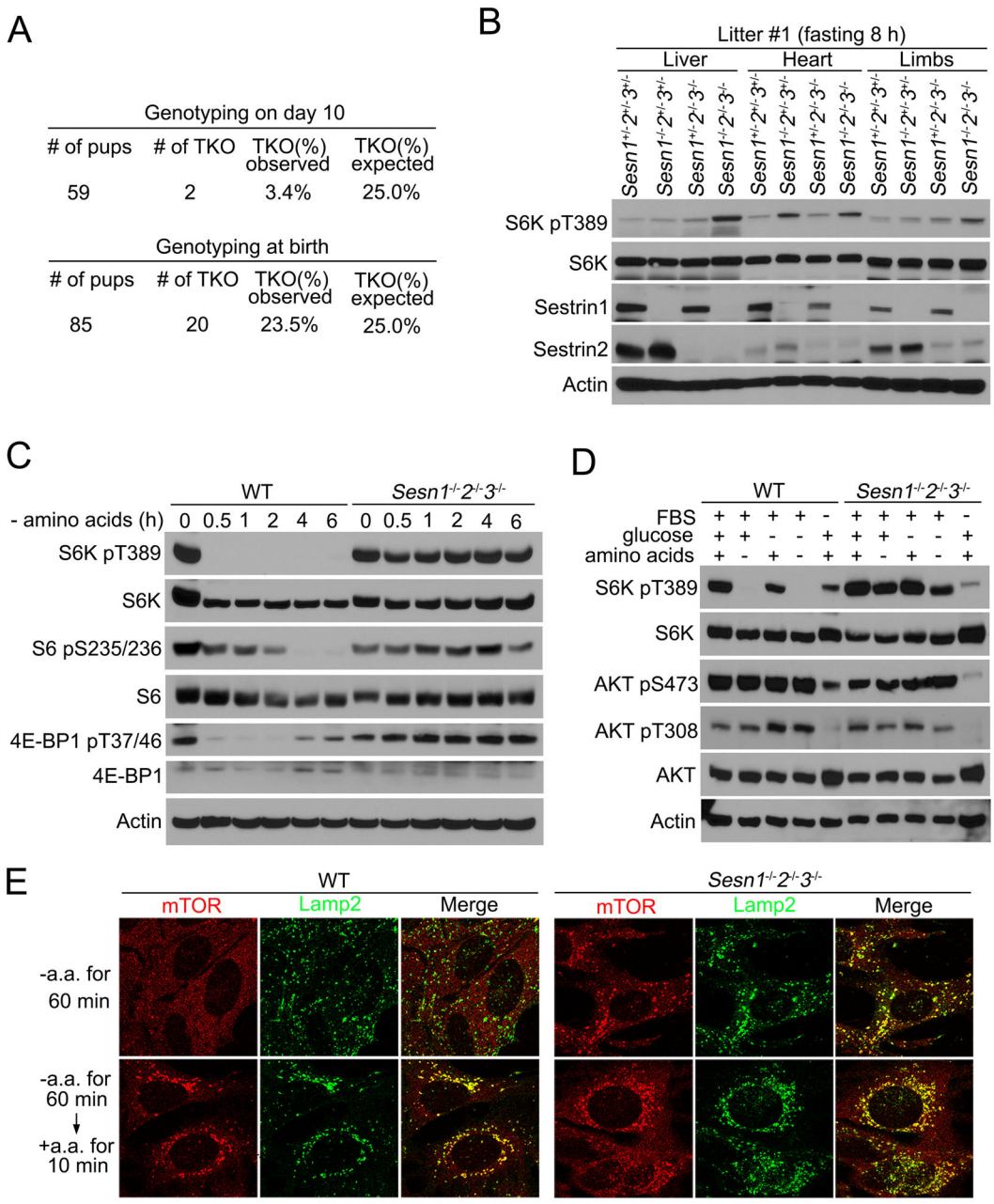

Figure 7. Sestrins Are Indispensable for Amino Acid Starvation-Triggered mTORC1 Inactivation

(A) Genotyping results of pups at 10 days after birth or neonates at birth.

(B) Total cell lysates prepared from the liver, heart or limbs of neonates with the indicated genotypes after 8 h of fasting were analyzed by immunoblotting.

(C) Wild-type (WT) and *Sesn1*$^{-/-}$*2*$^{-/-}$*3*$^{-/-}$ MEFs were starved for amino acids for 0.5, 1, 2, 4, and 6 h. Total cell lysates were analyzed by immunoblotting.

(D) WT and *Sesn1*$^{-/-}$*2*$^{-/-}$*3*$^{-/-}$ MEFs were starved for amino acids and/or glucose for 1 h, or FBS for 2 h. Total cell lysates were analyzed by immunoblotting.

(E) WT and *Sesn1*$^{-/-}$*2*$^{-/-}$*3*$^{-/-}$ MEFs were starved for amino acids for 60 min, and either left untreated or re-stimulated with amino acids for 10 min. The localization of mTOR and Lamp2 was determined by immunostaining.